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Relationship between airway colonization, inflammation and exacerbation frequency in COPD

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KEYWORDS
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Summary
Rationale: To evaluate bacterial colonization and the airway inflammatory response, and its relationship to the frequency of exacerbation in patients with stable chronic obstructive pulmonary disease (COPD).
Methods: Quantitative bacteriologic cultures, neutrophil elastase, myeloperoxidase (MPO), tumor necrosis factor alpha (TNF-α) and interleukin (IL)-8 were measured in bronchoalveoler lavage (BAL) in 39 patients with stable COPD [19 with frequent exacerbation ($\geqslant$3/year), and 20 with infrequent] and in 18 healthy controls (10 smokers and 8 non-smokers).
Results: BAL revealed the microorganisms with potential pathogenicity above the established threshold ($\geqslant 10^3$ cfu/ml) in 68.4% of patients with frequent exacerbation, 55% of infrequent exacerbation, 40% of smokers and 12.5% of non-smokers controls ($P = 0.05$). BAL MPO, IL-8 and TNF-α levels were found to be significantly higher in COPD as compared to controls ($P = 0.001$). However, only IL-8 level was significantly higher in COPD patients with frequent exacerbation as compared to infrequent ($P = 0.001$). Airway bacterial load correlated with levels of airway inflammation markers in COPD ($P < 0.05$).
Conclusion: The bacterial load and airway inflammation contributes to each other in stable COPD. However, there is a link only between interleukine (IL)-8 and frequent exacerbations. Clearly, the relationship between bacterial colonization, airway inflammation and frequent exacerbations is of major importance in understanding of the COPD pathogenesis.

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Introduction

Exacerbations of chronic obstructive pulmonary disease (COPD) are important causes of morbidity and mortality.[1–3] Some patients with COPD are particularly susceptible to develop frequent exacerbations,[4] which are now being recognized as an important feature of the natural history of COPD, with important implications for health related quality of life and hospital admission.[5]

The frequency of exacerbations increase with the severity of COPD, however, the mechanism modulating exacerbation frequency in patients with COPD are largely unknown. It has recently been shown that COPD patients with frequent exacerbations have higher sputum levels of IL-6 and IL-8 in the stable state than patients with infrequent exacerbations, suggesting that frequent exacerbations may be associated with increased airway inflammation.[6] Some recent data also indicate that lower airway bacterial colonization in the stable state modulates the character and frequency of COPD exacerbations.[4] However, definite conclusions about the exact role of airway inflammation and/or bacterial colonization in the modulating the exacerbation frequency in COPD are difficult to draw for a number of reasons. First, previous studies investigated only one aspect of the relationship between these factors: the relationship of frequency of exacerbations with airway inflammation or bacterial colonization. Furthermore, these factors are uncertain whether these represent cause or effect. Second, sputum was used in these studies instead of bronchoalveoler lavage (BAL), which gives a better assessment of small airways and alveolar inflammation and also airway bacterial colonization.[7,8] All of these may be insufficient to obtain a full view. The knowledge of this relationship may contribute to our further understanding of the pathogenesis of COPD.

In accordance, in the current study we aimed to concomitantly assess the relationships between frequency of exacerbation and airway inflammation as well as bacterial colonization by means of bronchoscopically retrieved BAL samples in patients with stable COPD.

Materials and methods

Study protocol

The study protocol was approved by the Ethical Committee of the Mersin University School of Medicine. Written informed consent was obtained from all subjects before enrolled into the study.

Study subjects

A prospective and observational study was undertaken. Thirty-nine subjects with clinically stable mild-to-moderate COPD, in accordance with GOLD guidelines[9] were included in the study. All COPD patients had a forced expiratory volume in 1 s (FEV_1)/forced vital capacity (FVC) ratio of $<70\%$ and reversibility of <200 ml or $<12\%$ in FEV_1 after inhalation of 200 μg salbutamol. All COPD patients were ex-smokers. As control group, 8 non-smoking and 10 smoking healthy, age-matched volunteers were studied. All control subjects had normal spirometric values. All subjects were assessed clinically and with a chest radiograph to ensure the absence of other significant respiratory disease. In addition, all COPD patients underwent high-resolution computed tomographic (HRCT) investigations to characterize the nature of their lung disease.

Study groups were as follows: Group I: Never smoker, healthy control subjects ($n = 8$), Group II: Current smoker, healthy control subjects ($n = 10$), Group III: COPD patients, with frequent exacerbation ($\geqslant 3$/previous year) ($n = 19$), and Group IV: COPD patients, with infrequent exacerbation (< 3/previous year) ($n = 20$).

Subjects with an exacerbation within the preceding month, a recent respiratory tract infection within the last 6 weeks, clinical history of asthma, atopy, allergic disease, bronchiectasis or other pulmonary disease, history of systemic disease or malignancies, inhaled or systemic corticosteroid therapy within 3 months and systemic antimicrobial therapy within the last 4 weeks before entry to the study were excluded.

Patients included in this study were selected from patients with COPD seen in our outpatient's clinic every 3 months over a 3 years period. Data relating to exacerbation numbers were obtained retrospectively from records of our outpatient's clinic. Exacerbations were diagnosed according to previous consensus definition, as follows, as a sustained worsening of the patient's condition from the stable state and beyond normal day-to-day variations that is acute in onset and may warrant additional treatment in a patient with underlying COPD.[10]

Pulmonary function and CO diffusing tests

The pulmonary function tests including the DL_{CO} measurements were performed in the plant using computer-assisted spirometry (Vmax22D, Sensor Medics, California, USA) in accordance with the European Respiratory Society's (ERS) recommendations. Measurements of FVC, FEV_1, FEV_1/FVC ratio, and maximal expiratory flow at 25–75% of VC ($FEF_{25-75}\%$) were obtained and expressed as percent predicted using normal values for adults of the European Community for Steel and Coal.[11] Carbon monoxide diffusing tests were performed by the single-breath method, also in accordance with ERS recommendations, to obtain a diffusing capacity of CO (DL_{CO} %).[12]

Bronchoscopy and BAL

Fiberoptic bronchoscopy including BAL was performed in all subjects. Prior to bronchoscopy, patients received premedication by intramuscular atropine (0.5 mg) and topical anesthesia by inhalation nebulized lignocaine (4 ml, 2% solution). Flexible bronchoscopy (Pentax EB-1830T3, Japan) was performed transnasally. After inspection the bronchial tree, the bronchoscope was wedged into a segmental bronchus of the right middle lobe and 5×20 ml sterile saline solution was infused. Fluid was gently aspirated immediately after the aliquot was introduced, the first reaspirated portion was discarded, and remaining BAL fluid collected in a sterile container. The total recovered BAL fluid was recorded, and then was strained through a

monolayer of surgical sterile gauze to remove mucus. The lavage sample was divided into three different aliquots for the cytological examination, measurements of cytokine and inflammatory mediators, and quantitative bacterial cultures. The sample for cytokine and inflammatory mediators was immediately centrifuged. Supernatants were removed and frozen at −70 °C until measuring for mediators.

Measurement of airway inflammatory markers

Airway inflammation was evaluated by means of measurements of neutrophil elastase (NE) and myeloperoxidase (MPO) activities, and IL-8 and tumor necrosis factor alpha (TNF-α) levels, and also albumin levels in BAL fluid.

BAL NE and MPO activity measurements: After BAL fluid samples were homogenized, NE and MPO measurements were performed. NE[13] and MPO[14] activity measurements were assessed spectrophotometrically. Absorbances were measured at 410 nm. Specific enzyme activity was calculated (Specific activity = Absorbance at 410 nm/g protein).

BAL IL-8 and TNF-α measurement: Solid-phase Sandwich Enzyme-Linked Immunosorbent Assay kits (Biosource International, CA, USA) were used for assaying IL-8 and TNF-α levels in the BAL supernatant. Measurements were made according to the kits procedure with automatic micro-ELISA machine (Tektime, BioMerieux, USA). All values were expressed as pg/ml. The minimum detectable concentrations were 5 pg/ml for IL-8 and 1.7 pg/ml for TNF-α.

Total protein measurement: Total protein content of BAL samples was measured according to the method of Lowry et al.[15]

Albumin measurement: BAL albumin was measured by colorimetric assay using a commercially kit (Spin React; Cod:1001020, lot: AL-72; Girona, Spain).

Permeability of the blood-airway lumen barrier: Plasma protein leakage into the airway lumen was analyzed by measuring albumin (Alb) in BAL fluid and paired serum samples.[16]

Microbiological evaluation

BAL samples were serially diluted (dilutions of 1:10, 1:100, and 1:1000). Diluted secretions were plated on blood, chocolate, eosin–methylene and Sabouraud agar. Some samples of BAL fluid were centrifuged and stained by the Gram and Giemsa method. Additionally, epithel and neutrophil counts and morphology of microorganisms were evaluated. Identification of microorganisms was performed according to standard methods.[17] Results of quantitative cultures were expressed in colony-forming units per milliliter (cfu ml). Bacterial agents were classified into potentially pathogenic microorganisms (PPMs) or non-PPM.[18,19] In order to minimize the risk of dealing with contaminants, only microorganisms with counts of $\geqslant 10^3$ cfu ml^{-1} in BAL fluid were regarded as significant.

Statistical analysis

Data analyses were performed with the Statistical Package for the Social Science (SPSS 11.5, California, USA). Data are expressed as means ± standard deviation. χ^2 Tests were used to compare the differences in the characteristics of two groups. Data of independent two groups were compared using Student *t*-test, whereas data of more than two groups compared using one-way analysis of variance (ANOVA) followed by Tukey HSD posthoc test to determine statistical differences. Comparisons of two proportions between the study and control groups were performed using the *z*-test. Additionally, repeatability test was performed for serologic measurements. Correlations were calculated by Spearman correlation coefficient. Binary logistic regression analysis (with Forward LR method) was performed to determine the significant risk factors, which have a role for the increasing in the exacerbation frequency. Statistical significance was considered at $P<0.05$ level.

Results

Patients

Demographic characteristics of controls and patients with COPD are given in Table 1. There were no statistical differences among groups with regard to age, sex, cigarette consumption or body mass index (BMI) (Table 1).

The mean predicted FEV_1 was 70.6 ± 12%, with a range from 50% to 88%. Therefore, all COPD patients can be classified as suffering from mild-to-moderate COPD according to GOLD criteria.[14] Functional data of controls and COPD patients are summarized in Table 2.

Bronchoscopy was well tolerated by all subjects without adverse effects.

During BAL procedure, the mean recovery of the injected fluid was similar among groups, and found to be 37.9 ± 6.5 ml in COPD patients with frequent exacerbation, 38.7 ± 11 ml in COPD patients with infrequent exacerbation, 40.7 ± 10.3 ml in the smokers control subjects, 41.6 ± 12.7 ml the non-smokers control group ($P>0.05$).

Results of airway inflammatory markers

NE and MPO activities of BAL were found to be higher in the COPD patients with frequent exacerbations as compared to COPD patients with infrequent exacerbations, but the difference was not statistically significant (Fig. 1a and b).

BAL IL-8 and TNF-α levels were found to be higher in the COPD patients with frequent exacerbations as compared to COPD patients with infrequent exacerbations, but the difference was statistically significant for only IL-8 levels ($P = 0.001$) (Fig. 1c and d).

Serum/BAL albumin leakage was 1.1 ± 0.9 in COPD patients with frequent exacerbation, 0.89 ± 0.3 in patients with infrequent exacerbations, 0.41 ± 0.3 in the smokers control group and 0.29 ± 0.5 in the non-smokers control group ($P = 0.001$).

Microbiological results

BAL revealed the presence of microorganisms with potential pathogenity above the established threshold ($\geqslant 10^3$ cfu/ml) in 13 (68.4%) of COPD patients with frequent exacerbations, in 11 (55%) of COPD patients with infrequent exacerbations,

Table 1 Characteristics of controls and patients with COPD.

	Control		COPD	
	Non-smoker	Smoker	Frequent exacerbation	Infrequent exacerbation
Subjects (*n*)	8	10	19	20
Sex				
Male	6	9	17	18
Female	2	1	2	2
Age*	45.6±4.9	50.2±4.5	58.84±7.7	58.55±7.7
BMI (kg/m^2)	26.92±4.3	27.7±4.9	23.63±3.9	25.47±4.5
Cigarette consumption (pack/years)*	—	37.7±13.3	50.26±22.2	46.20±22.1
Number of exacerbation (previous year)*	—	—	5.73±4.1	1.1±0.8

*(Mean±SD).

Table 2 Pulmonary function tests and arterial blood gases.

	Control		COPD	
	Non-smoker	Smoker	Frequent exacerbation	Infrequent exacerbation
FVC (% predicted)	109.6±8.7	99.2±17.7	89.1±13.5	96.7±12.9
FVC (l)	4.5±1.4	3.9±0.7	3.3±0.7	3.6±0.7
FEV_1 (% predicted)	107.1±6.3	95±16.2	65.8±12.8	70.5±12
FEV_1 (l)	3.6±0.9	3.1±0.,5	1.9±0.5	2.1±0.5
FEV_1/FVC	81±5.2	78.5±4.1	58.5±7.2	60.7±4.7
$FEF_{25–75}$ (% predicted)	96.3±19.8	75.3±18.1	25.1±8.6	32.8±9.9
DL_{CO} (% predicted)	104.3±24	107.2±13.2	80.4±26.8	85.8±32.1
SaO_2 (%)	97.4±1.1	96.8±1.3	95.3±1.4	95.7±1.1
PaO_2 (mmHg)	94.6±10.6	86±8.5	78.6±8.4	81.7±6.9
$PaCO_2$ (mmHg)	37.3±3.8	37±2.3	38.2±3.6	36.5±3.6
pH	7.42±0.38	7.42±0.01	7.40±0.02	7.41±0.02
HCO_3	24±1.6	23.4±1.1	23.7±1.9	23±1.9

Data were presented as mean±SD.

and in 4 (40%) of smokers controls and in 1 (12.5%) of non-smokers controls ($P = 0.05$). Overall colonization rates were 61.5% (24 out of 39) in all COPD patients and 27.8% (5 out of 18) in all controls ($P = 0.018$) (Table 3). Total 9 of 24 colonized COPD patients, 4 of the 13 colonized COPD patients with frequent exacerbation and 5 of the 11 colonized COPD patients with infrequent exacerbations, had multiple organisms. Three COPD patients had triple infection, and 6 had double. *Neisseria* species being the most common microorganism isolated followed by both *Streptococcus* spp., and *Haemophilus influenzae* in multiple infections of COPD patients.

The mean BAL neutrophil cell count was found to be higher in COPD patients with frequent and infrequent exacerbation as compared to non-smoking controls (Fig. 2).

COPD patients have significantly higher total bacterial load as compared to the controls ($P = 0.017$). However, there was no statistically significant difference between COPD patients with frequent exacerbations and patients with infrequent exacerbations with respect to total bacterial load (Fig. 3).

The relationship of bacterial colonization, airway inflammatory markers and exacerbation frequency

Bacterial load were positively correlated with airway inflammatory markers in patients with COPD. Correlation coefficient was strong especially for IL-8 ($r = 0.52$, $P = 0.009$) (Fig. 4).

In subjects with COPD, increased serum/BAL albumin leakage was strongly correlated with increased BAL neutrophil counts ($r = 0.47$, $P = 0.02$) and airway bacterial load ($r = 0.41$, $P = 0.03$). These findings suggest a possible causal relationship between lung vascular permeability and neutrophil numbers in subjects with COPD.

Logistic regression analysis was performed to determine the significant predictive factors for the frequent/infre-

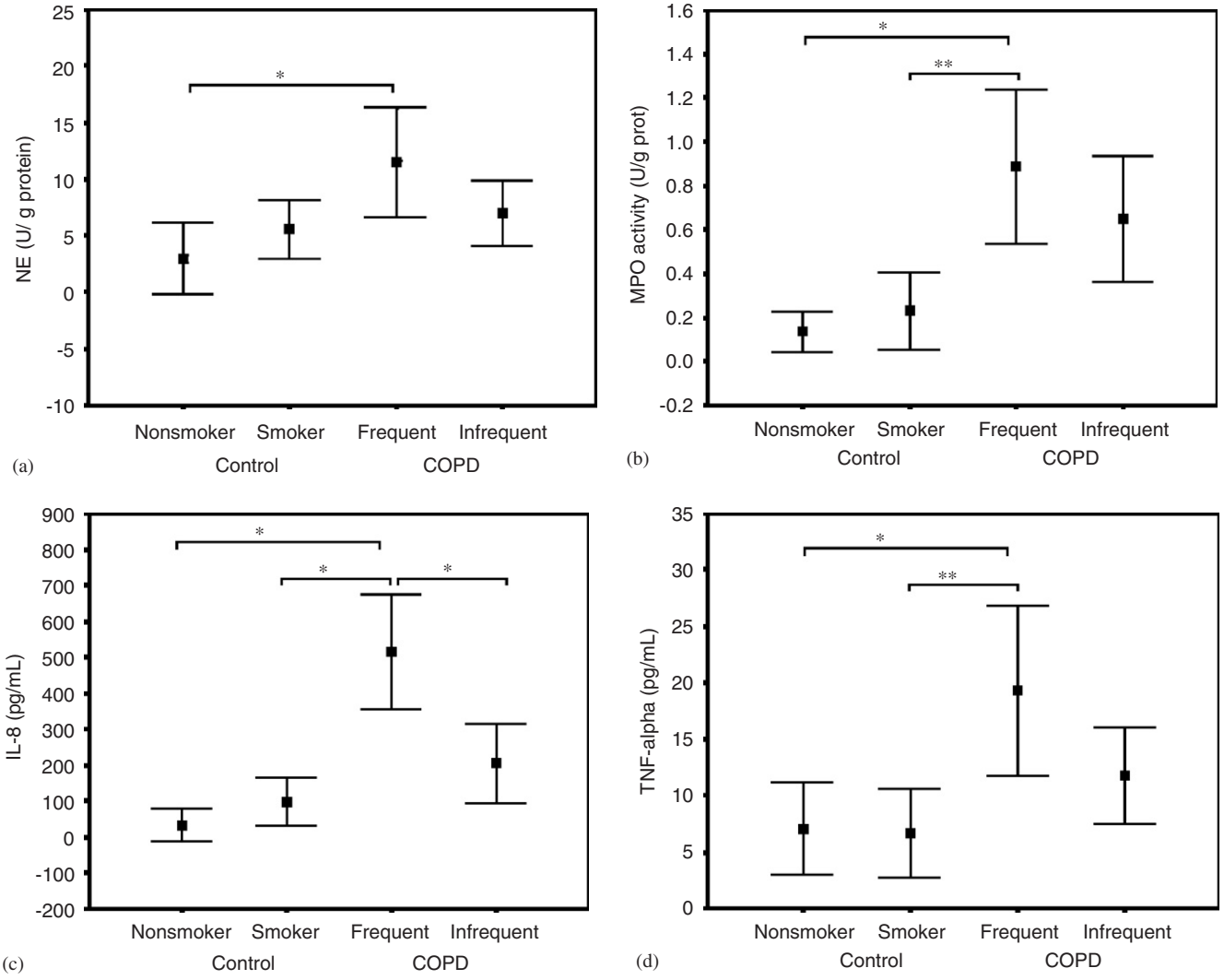

Figure 1 Levels of inflammatory markers in BAL fluid. Horizantal bars represent mean $\pm$ 95% confidence interval values: (a) NE activity ($P = 0.028$ with ANOVA, and $^{*}P = 0.034$ with Tukey posthoc test), (b) MPO activity ($P = 0.005$ with ANOVA, and $^{*}P = 0.015$, $^{**}P = 0.024$ with Tukey posthoc test), (c) IL-8 levels ($P = 0.001$ with ANOVA, and $^{*}P = 0.001$ with Tukey posthoc test) and (d) TNF-α levels ($P = 0.012$ with ANOVA, and $^{*}P = 0.05$, $^{**}P = 0.024$ with Tukey posthoc test).

Table 3 Isolated microorganisms in BAL fluid ($\geqslant 10^3$).

	Control ($n = 18$)		COPD ($n = 39$)	
	Non-smoker ($n = 8$)	Smoker ($n = 10$)	Frequent exacerbation ($n = 19$)	Infrequent exacerbation ($n = 20$)
Streptococcus pneumoniae	0	0	0	2
Haemophilus influenzae	0	0	2	0
Moraxella catarrhalis	0	0	1	0
Staphylococcus aureus	0	0	1	0
Streptococcus spp.	1	4	10	10
Neisseria species	0	0	3	2
Coagulase (−) *Staphylococcus*	0	0	0	1
Haemophilus spp.	1	0	0	1
Total*	1 (12.5%)	4 (40%)	13 (68.4%)	11 (55%)

*Total number of subjects in whom at least one microorganism was isolated in BAL.

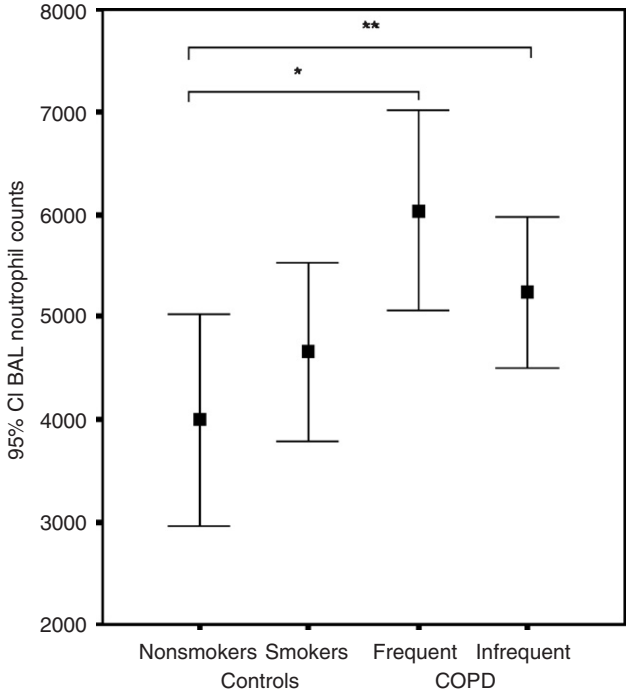

Figure 2 Neutrophil counts in BAL fluid. Neutrophil count values represent the number of neutrophil cells $\times$ ml^{-1} of recovered lavage fluid. Horizontal bars represent mean $\pm$ 95% confidence interval values ($P = 0.025$ with ANOVA, and $^{*}P = 0.026$, $^{**}P = 0.044$ with Tukey posthoc test).

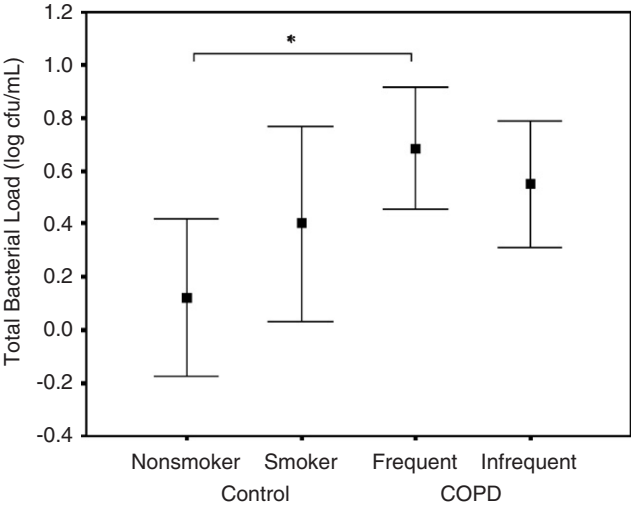

Figure 3 Total bacterial load in BAL fluid. Horizontal bars represent mean $\pm$ 95% confidence interval values ($P = 0.049$ with ANOVA, and $^{*}P = 0.039$ with Tukey posthoc test).

quent exacerbations in COPD patients. This analysis showed that 1 pg/ml of increase in BAL IL-8 level increased the risk of frequent exacerbations 1 fold ($P = 0.022$, Exp $B = 1.007$, CI %95: 1.001–1.012), and per hundred milliliter decreases in FEV_1 increased the risk of frequent exacerbations 1 fold ($P = 0.05$, Exp $B = 0.099$, CI %95: 0.010–1.012), when the exacerbation frequency was the dependent variable, and smoking, total bacterial load, FEV_1 and airway inflammatory

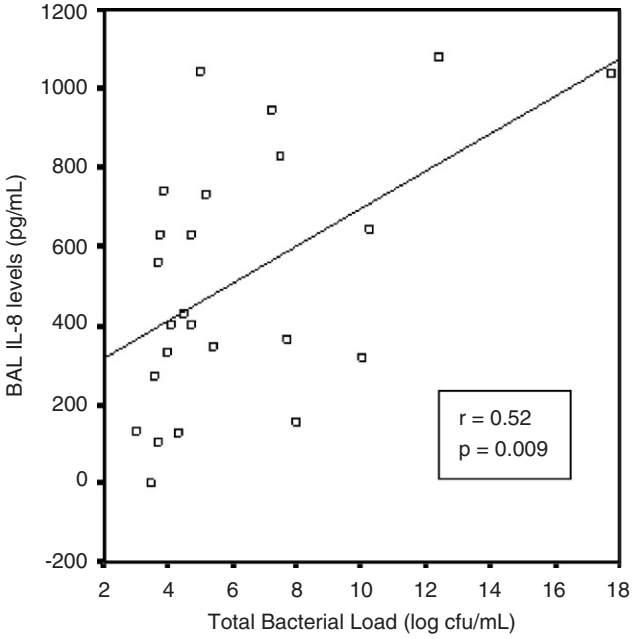

Figure 4 Correlation between BAL total bacterial load and BAL IL-8 levels in COPD patients. Total bacterial load were positively correlated with IL-8 levels ($r = 0.52$, $P = 0.009$).

markers were independent variables. There was not a significant relationship between airway colonization and exacerbation frequency.

Discussion

In the present study, we found that subjects with COPD who do not currently smoke have airways inflammation as shown by a significant increase in IL-8, TNF-α and MPO levels, and protein leakage, and total bacterial load compared with healthy controls. Also, airway bacterial load was positively correlated with inflammation markers in BAL in patients with COPD. Among airway inflammatory markers, there is a link only between IL-8 and frequent exacerbations in COPD patients. However, a significant relationship was not found between airway colonization and exacerbation frequency in COPD patients.

COPD is characterized predominantly by neutrophilic airway inflammation and this is thought to represent an exaggerated response to tobacco smoking. Several studies showed increased IL-8 levels in induced sputum and BAL in patients with COPD compared with smokers without COPD.[20–22] There is also evidence that bacterial products affect neutrophil migration and may independently modulate airway inflammation.[23,24] Bacterial colonization of distal airways is frequently found in patients with stable COPD.[4,24,25] Reported bacterial colonization rate in stable COPD patients changes from 33% to 100%. Important causes of this wide range are using different airway samples (sputum, protected brush, BAL) and the risk factors influencing bacterial colonization (smoking, airway obstruction severity, airway inflammation).[26] In this study, we evaluated airway colonization using BAL samples. Accordingly, overall airway bacterial colonization rates were 61.5%

(24 out of 39) in all COPD patients and 27.8% (5 out of 18) in all controls. Some patients (total 9 of 24 colonized COPD patients) are infected two or more microorganism. *Neisseria* species being the most common microorganism isolated followed by both *Streptococcus* spp., and *H. influenzae* in multiple infections of COPD patients. The bacterial colonization rate and bacterial species that we isolated are similar to those found in many prior studies of sputum and lower airway bacteriology.[26] The significance of bacterial colonization of the bronchial tree in terms of the development and progression of airway inflammation is not precisely known. Nevertheless, there is evidence that inflammatory response to bacterial pathogens is an important contributor to the damage of epithelial surfaces, thus promoting further bronchial colonization.[4,25,27] Soler et al.[25] showed that bacterial colonization in the distal airways is associated with increased polymorphonuclear cells and TNF-α in BAL fluid from patients with COPD. Patel et al.[4] found that the bacterial count was related to the sputum level of IL-8. Hill et al. demonstrated that increasing airway bacterial load was strongly related to several markers of inflammation in airway secretions, including sputum MPO and IL-8 levels, as well as leukocyte elastase activity.[16] In the present study, we have again confirmed that higher airway bacterial load is associated with greater airway inflammation in terms of BAL IL-8, TNF-α and MPO levels and also protein leakage in patients with COPD. Additionally, in subjects with COPD, increased serum/BAL albumin leakage was strongly correlated with increased BAL neutrophil counts. These findings suggest a possible causal relationship between lung vascular permeability and neutrophil numbers in subjects with COPD, and that the bacterial load and airway inflammation contributes to each other in patients with stable COPD.

Recent data indicates that some patients with COPD develop frequent exacerbations, and recurrent exacerbations may be associated with increased airway inflammation, although it is uncertain whether this represents cause or effect. Bhowmick et al.[28] showed that patients with frequent exacerbations had heightened airway inflammation when stable as measured by induced sputum IL-6 and IL-8 levels. The interpretation of these results is far from clear as many factors may influence inflammation in the airways. These include the severity of airflow obstruction, smoking status, bronchiectasis, bacterial colonization, treatment with inhaled corticosteroids, and the exacerbation itself.[6] Although, Bhowmick et al.[28] postulated that this might be due to the load of bacteria in the lower airways, there was no information concerning current smoking status, bacterial colonization or use of inhaled corticosteroids, all of which might have influenced the results. It is possible that frequent exacerbations may have a continuing influence on bronchial inflammation even in the stable clinical state. On the other hand, in the study of Gompertz et al.[6] in which the relationship between airway inflammation and the frequency of exacerbations was examined in patients with COPD, authors found no differences in any of inflammation markers including IL-8 between the patients with frequent and infrequent exacerbations. There were also no difference in the severity of airflow obstruction, current smoking status, or in the prevalence or degree of airway colonization, all of which are known to influence airway inflammation. They concluded that there are several clinical features that directly influence airway inflammation in COPD, and when these were carefully controlled for patients with more frequent reported exacerbations did not have significantly greater bronchial inflammation.[6] In the present study, among airway inflammatory markers, there is a link only between IL-8, a neutrophil chemoattractant, and frequent exacerbations in COPD patients. The difference in the results of inflammation or colonization between the different studies may be related to several unreported, undetected, or uncontrolled factors for clinical differences between the groups of patients. Thus, we did not include subjects with bronchiectasis or inhaled or systemic corticosteroid therapy in the study in order to study airway inflammation and colonization of COPD as well as possible. Previous studies have indicated that, although clinically indistinguishable, patients with bronchiectasis have evidence of increased airway inflammation and colonization, and airway inflammation and colonization may be altered by the nature of the disease (COPD or bronchiectasis).[29] One of the other well-known confounding factor that influence the airway inflammation, colonization and/or exacerbation frequency, is steroid using. Although, inhaled corticosteroids do not modify disease progression in COPD, there is increasing evidence that they reduce the number of exacerbations in COPD.[30] Corticosteroids are also known to have some beneficial effects on bronchial inflammation, including reducing airway neutrophilia[31] and reducing protein leakage.[6] On balance, therefore, it is likely that steroids do alter airway inflammation and again this should be born in mind when assessing the results of patient studies. Moreover, the result of most studies indicated that inhaled steroids were prescribed to patients with higher inflammatory activity, thus, this factor could effect the difference in COPD patients as compared to controls.[6,25] In this study, additionally, logistic regression analysis was performed to determine the other possible significant risk factors which had a role in the increasing in the exacerbation frequency in COPD patients. Logistic regression analysis of the data from this study showed that BAL IL-8 level and severity of airflow obstruction were predictive significant risk factors for frequent exacerbation, however, it did not show a significant influence of smoking, total bacterial load, and airway inflammatory markers except IL-8 on exacerbation frequency. Patel et al.[4] found that the presence of bacterial colonization in the stable state was associated with increased exacerbation frequency, and patients with colonization had longer and thus more severe exacerbations. In the present study, unlike as Patel et al.,[4] we did not find a significant relationship between airway colonization and exacerbation frequency in COPD patients. This might be a surprising result. As we found that patients with a history of frequent exacerbations had increased airway inflammation in terms of BAL IL-8 level, and airway bacterial load were positively correlated with inflammation markers in BAL, bacterial colonization might be expected to be an important factor for exacerbation frequency.

We believe that the superiority of our study is that it examines BAL, while most previous studies have examined sputum.[4,6,25,26] BAL should give a better assessment of small airways and alveolar inflammation, and also more reliable for assessment of airway bacterial colonization than sputum.[7,8] However, generally, there are some problems

with the assessment of bacterial colonization or protein of BAL fluid samples. There is a risk of contamination of bacteria during the FOB and BAL procedure, because some bacteria species are normal naso-oro-pharyngeal flora, non-quantitative culture may be confusing, because it cannot confirm a lower respiratory source for these isolates. In order to minimize the risk of dealing with contaminants, we used quantitative cultures of BAL fluid samples as the gold standard, and only microorganisms with counts of $\geqslant 10^3$ cfu ml^{-1} in BAL fluid were regarded as significant. An another problem with BAL fluid samples is the recovery and dilution of sample material. The analysis of protein levels in BAL fluid is complicated by variable dilution of the epithelial lining fluid (ELF) by the BAL procedure, which may differ between patients and within patients. One approach to take into account the variable dilution, proposed by some authors, is to use a correction factor based on concentrations of a reference protein (e.g. albumin or urea) in serum and BAL fluid to calculate protein levels in ELF.[32] It is also reported that in the analysis of lung proteins recovered by lavage, some of recovered proteins depend on lavage volume. Larger volumes will add more protein but not alter protein ratios.[33] In this study, total protein content of BAL fluid was measured, and used as a marker of ELF dilution allowing the calculation of an apparent ELF volume and adjusted protein content. To our best knowledge, this is the first study concomitantly assessing the relationship between the frequency of exacerbation and airway inflammation as well as bacterial colonization by means of bronchoscopically retrieved BAL samples in patients with stable COPD. Our study enriched the findings of two previous studies using sputum samples in their evaluations; the first is the study of Gompertz et al.,[6] which investigated the relationship between exacerbation frequency and bronchial inflammation, and the second is the study of Patel et al.,[4] which evaluated the relationship between bacterial colonization and the exacerbation frequency in COPD patients.

In conclusion, the bacterial load and airway inflammation contributes to each other in patients with stable COPD. However, there is a link only between IL-8 and frequent exacerbations. Clearly, the relationship between bacterial colonization, bronchial inflammation and frequent exacerbations is of major importance in understanding of the pathogenesis of COPD.

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